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# Catalase, Superoxide Dismutase, and Hemolysin Activities and Heat Susceptibility of *Listeria monocytogenes* after Growth in Media Containing Sodium Chloride

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**The activities of catalase, superoxide dismutase, and a thiol-activated hemolysin produced by four strains of *Listeria monocytogenes* propagated in media containing various concentrations of sodium chloride were examined. *L. monocytogenes* 7644 showed an increase in catalase, superoxide dismutase, and thiol-activated hemolysin activities when grown in a medium containing 2.5% (wt/vol) NaCl followed by a decrease in activities when propagated in media containing salt concentrations higher than 2.5%. *L. monocytogenes* LCDC 81-861 demonstrated enhanced catalase activity when grown in media containing NaCl ranging from 1.5 to 4.6% and increased superoxide dismutase activity when propagated in media containing 1.5 to 3.5% NaCl. *L. monocytogenes* LCDC 81-861 did not exhibit any detectable hemolysin activity under the conditions tested. After growth in various NaCl-containing media, both strains were subjected to sublethal heat injury for 30 min at 55°C. *L. monocytogenes* LCDC 81-861 showed increased sensitivity to the heat treatment when grown in media containing 4.6 and 6.5% NaCl, whereas *L. monocytogenes* 7644 did not exhibit enhanced heat lability.**

*Listeria monocytogenes* is a gram-positive, aerobic to facultatively anaerobic, intracellular pathogen that has recently been cited in several food-borne disease outbreaks (10). *L. monocytogenes* is a salt-tolerant bacterium capable of surviving for 8 weeks in 20% NaCl at 4°C (24). Shahamat et al. (25) found that the organism could survive for 15 days in media containing 10.5% NaCl and 5 days in media containing 20 to 30% NaCl when the temperature was 37°C. McClure et al. (20) discovered that *L. monocytogenes* would grow in media containing 10% NaCl (pH 7.0) at 25°C.

Catalase (CA), superoxide dismutase (SOD), and a thiol-activated hemolysin (TAH) are among the suggested virulence factors of *L. monocytogenes* (2, 18). Toxic oxygen species such as the superoxide radical ($O_2^-$), hydrogen peroxide ($H_2O_2$), and the hydroxyl radical (OH · ) are produced during the respiratory burst in macrophages and neutrophils. CA and SOD are two listerial enzymes that detoxify these oxygen species. The *L. monocytogenes* hemolysin is a pore-forming cytolysin similar in antigenicity to streptolysin O (26). Recently it was shown that nonhemolytic mutants of *L. monocytogenes* are avirulent (6, 14).

There is a paucity of information available about how the environmental conditions in which *L. monocytogenes* is grown affect its virulence. Sodium chloride is used in many food systems as an antimicrobial agent. Junttila and Brander (12) reported an *L. monocytogenes* septicemia in an elderly man who had eaten salted mushrooms stored for 5 months at cold temperatures. The purpose of this study was to determine CA, SOD, and TAH activities when *L. monocytogenes* was grown in media containing various levels of NaCl. Also, heat susceptibility was investigated for cells propagated in salt-containing media.

## MATERIALS AND METHODS

**Organisms and growth conditions.** *L. monocytogenes* ATCC 7644 and ATCC 15313 were obtained from American Type Culture Collection, Rockville, Md.; strains LCDC 81-861 and Scott A were obtained from Larry Beuchat, University of Georgia Experimental Station, Experiment, Ga.

Frozen stock cultures were prepared by inoculating 10 ml of tryptic soy broth (TSB) with 0.1 ml of an overnight stationary-phase inoculum, vortexing the two, and freezing the mixture at −20°C. *L. monocytogenes* LCDC 81-861 frozen stocks were thawed and inoculated into 250-ml Erlenmeyer flasks containing either TSB, TSB with 1.5% (wt/vol, final concentration) NaCl (TSBS 1.5), TSB with 2.5% (final concentration) NaCl (TSBS 2.5), TSB with 3.5% (final concentration) NaCl (TSBS 3.5), TSB with 4.6% (final concentration) NaCl (TSBS 4.6), or TSB with 6.5% (final concentration) NaCl (TSBS 6.5). *L. monocytogenes* strains 7644, 15313, and Scott A frozen stocks were thawed and inoculated into 250-ml Erlenmeyer flasks containing either TSB, TSBS 2.5, TSBS 4.6, or TSBS 6.5. Cultures of 100 ml were grown at 37°C on a gyratory shaker (120 rpm) to the stationary phase as determined by monitoring with a Klett-Summerson photoelectric colorimeter (Klett Manufacturing Co., New York, N.Y.).

**Preparation of cell extracts for CA and SOD assays.** Early-stationary-phase cultures were harvested by centrifugation (16,300 × *g*, 10 min) and washed in 50 mM potassium phosphate buffer (pH 7.0). Cell extracts were prepared by passing cells through a French pressure cell (American Institute Corp., Silver Spring, Md.) at a pressure of 7,600 lb/in$^2$. The resulting lysate was centrifuged at 12,000 × *g* for 10 min at 4°C, and the supernatant fluid was retained on ice.

**CA and SOD assays.** CA activity was determined by the spectrophotometric method of Beers and Sizer (1). One unit of CA decomposes 1.0 μmol of $H_2O_2$ per min at 25°C at pH 7.0, whereas the $H_2O_2$ concentration falls from 10.3 to 9.2 μmol/ml of reaction mix.

SOD activity was measured by the cytochrome *c* reduction method of McCord and Fridovich (21). One unit of SOD is defined as the amount required to inhibit the rate of reduction of cytochrome *c* by 50%.

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The standard error of the mean was determined by entering specific activities of CA and SOD into a Sigma-Plot graphics program (Jandel Scientific, Sausalito, Calif.).

**TAH assay.** Hemolysis was detected by a modification of the procedure of Cowart and Foster (3). Early-stationary-phase cultures were centrifuged (12,000 × *g*, 4°C), and the crude supernatant fluid was retained. Various dilutions of the supernatant fluid were made in 10 mM potassium phosphate buffer (pH 6.6) containing 130 mM NaCl and 6 mM cysteine. One milliliter of freshly washed sheep erythrocytes (1.5%, vol/vol) was added to 2 ml of the diluted supernatant fluid series. After the erythrocytes were added, the tubes were immediately shaken and placed in a 37°C water bath for 1 h. After incubation the reaction mix was centrifuged (800 × *g*), and the supernatant fluid was transferred into 1-ml cuvettes. The $A_{530}$ was measured in a Beckman model DU-40 spectrophotometer. The $A_{530}$ of 100% hemolysis was determined by the addition of 1 ml of erythrocyte suspension in 2 ml of water. The TAH titer was quantitated by determining the 50% endpoint as described by Garvey et al. (8). Linear regression analyses were performed on the data, and correlation coefficients were calculated. Protein concentrations of the supernatant fluids were determined to normalize the TAH titers. The standard error of the mean was determined by entering the TAH titers into the Sigma-Plot graphics program.

**Protein assay.** Protein concentrations of the lysates and supernatant fluids were determined by the method of Lowry et al. (19) with lysozyme as the standard.

**Heat injury.** *L. monocytogenes* 7644 and LCDC 81-861 were grown in TSB, TSBS 2.5, TSBS 4.6, and TSBS 6.5 at 37°C to obtain stationary-phase cultures. The cells were pelleted by centrifugation (16,300 × *g*, 4°C) and suspended in an equal volume of 100 mM potassium phosphate buffer (pH 7.2). Cells were plated to determine CFU per milliliter on tryptic soy agar (TSA) and TSA with 5.5% NaCl final concentration (TSAS). Heat injury proceeded as described by Dallmier and Martin (4) for 30 min at 55°C with stirring. After heating, cells were diluted in 0.1% sterile peptone water and spread plated in triplicate on TSA and TSAS. Enumeration on TSA, a nonselective medium, represented the total CFU per milliliter; enumeration on TSAS, a selective medium, represented the number of uninjured cells. The difference between the two counts was defined as the number of injured cells.

## RESULTS

**CA activity.** The CA activity of *L. monocytogenes* 7644 cells grown in TSBS 2.5 increased compared with that of cells grown in TSB (Fig. 1). With the salt concentrations higher than 2.5%, there was a decline in CA activity, resulting in a lower CA activity for cells cultured in TSBS 6.5 in comparison with that of cells propagated in TSB. The activity of *L. monocytogenes* 15313 grown in identical NaCl concentrations was similar to that of strain 7644 (data not presented).

*L. monocytogenes* LCDC 81-861, a high-CA-producing strain, exhibited a CA activity for cells grown in 3.5% NaCl that was approximately 140% that of cells cultured in TSB (Fig. 2). Cells cultured in media containing NaCl concentrations higher than 3.5% demonstrated decreasing CA levels, resulting in a CA activity for cells grown in TSBS 6.5 that was lower than the CA activity for cells grown in TSB. *L. monocytogenes* Scott A and LCDC 81-861 showed similar results when grown in identical NaCl levels (data not presented).

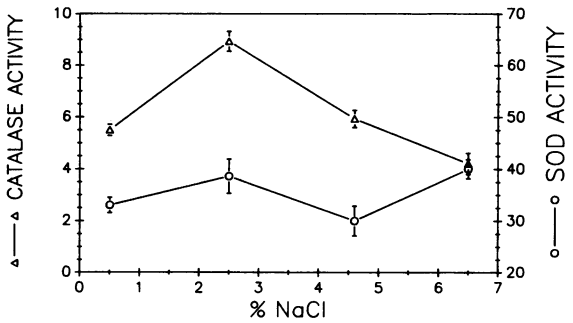


FIG. 1. CA and SOD specific activities (units per milligram of protein) of *L. monocytogenes* 7644 after growth in TSB containing various levels of NaCl. Error bars represent the standard errors of the means.

**SOD activity.** *L. monocytogenes* 7644 demonstrated fluctuating SOD activities when cultured in media containing increasing NaCl concentrations (Fig. 1). When strain 7644 was grown in TSBS 2.5, SOD activity increased in comparison with that of cells grown in TSB. After a drop in SOD activity of cells grown in TSBS 4.6, there was an increase in SOD activity for cells propagated in TSBS 6.5 compared with that of TSB-cultured cells. *L. monocytogenes* 15313 showed increasing SOD levels when grown in media containing NaCl concentrations higher than 2.5%, giving an SOD level higher for TSBS 6.5-cultured cells compared with that of TSB-cultured cells (data not shown).

*L. monocytogenes* LCDC 81-861 had an increase in SOD activity when grown in media containing 1.5% NaCl compared with that of cells grown in TSB (Fig. 2). At NaCl concentrations ranging from 1.5 to 3.5%, there was a gradual decrease in SOD activity, followed by a sharp drop when the NaCl concentration was greater than 3.5%. Eventually there was a decrease in SOD activity for cells grown in TSBS 6.5 compared with that for cells grown in TSB. *L. monocytogenes* Scott A exhibited a gradual decline in SOD levels when grown in increasing NaCl concentrations, resulting in a lower SOD activity in TSBS 6.5 compared with that of cells grown in TSB (data not shown).

**Heat injury.** The effect of growth in NaCl-containing media on the heat resistance of *L. monocytogenes* 7644 and LCDC 81-861 is shown in Table 1. The growth conditions had little effect on the heat resistance of *L. monocytogenes* 7644 when the counts of heated cells recovered on TSA and counts of heated cells recovered on TSAS were compared.

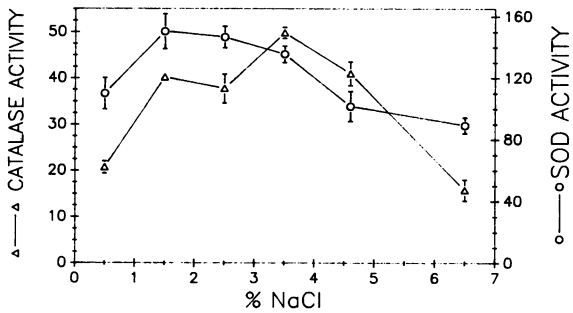


FIG. 2. CA and SOD specific activities (units per milligram of protein) of *L. monocytogenes* LCDC 81-861 after growth in TSB containing various levels of NaCl. Error bars represent the standard errors of the means.

TABLE 1. Counts produced by cultures of *L. monocytogenes* cells grown in TSB containing NaCl after heat injury at 55°C in buffer

| % NaCl in medium | Log CFU of 7644 per ml | | | | Log CFU of LCDC 81-861 per ml | | | |
|---|---|---|---|---|---|---|---|---|
| | Unheated cells | | Heated cells | | Unheated cells | | Heated cells | |
| | TSA | TSAS | TSA | TSAS | TSA | TSAS | TSA | TSAS |
| 0.5 | 9.9 | 9.7 | 6.1 | 3.1 | 10.1 | 9.6 | 6.5 | 3.8 |
| 2.5 | 9.9 | 9.4 | 7.1 | 4.2 | 9.6 | 9.4 | 7.0 | 4.4 |
| 4.6 | 9.7 | 9.3 | 7.4 | 4.5 | 9.1 | 9.1 | 7.7 | 3.2 |
| 6.5 | 9.0 | 8.9 | 6.6 | 4.0 | 8.5 | 8.4 | 7.5 | 2.8 |

*L. monocytogenes* LCDC 81-861 grown in TSB and TSBS 2.5 showed heat resistance similar to that of *L. monocytogenes* 7644. However, when *L. monocytogenes* LCDC 81-861 was grown in either TSBS 4.6 or TSBS 6.5, the heat sensitivity increased.

**TAH production.** TAH titers from *L. monocytogenes* 7644 grown in media containing various NaCl concentrations are shown in Fig. 3. Like CA and SOD activities, TAH activity of cells grown in TSBS 2.5 was stimulated compared with that of cells grown in TSB. Titers of cultures propagated in TSBS 4.6 were approximately the same as those of cells grown in TSB. A decrease in TAH titer was found for cells grown in TSBS 6.5. *L. monocytogenes* 15313, LCDC 81-861, and Scott A did not exhibit any detectable TAH activity in any of the salt concentrations tested (data not shown).

## DISCUSSION

Very little work has been done concerning the effects of NaCl on enzyme biosynthesis and/or activity in salt-tolerant bacteria such as *L. monocytogenes*. Most of the research in this area has been performed on salt-dependent enzymes in halophilic archaebacteria (17) and halophilic eubacteria (13). Galligan et al. (7) found increased CA activities for *Staphylococcus aureus* MF-31 cultures grown in TSB containing 1.5 to 3% NaCl. When *S. aureus* MF-31 was grown in media containing salt concentrations above 3.0%, the CA activity decreased sharply, falling below the CA activity for cells cultured in media with 0.5% NaCl. Udou and Ichikawa (28) discovered that *S. aureus* cells grown in media containing 0.6 or 3% NaCl had enhanced oxygen consumption. However, when the cells were cultured in a medium with 6% NaCl, oxygen consumption was reduced. This increase in oxygen consumption could lead to increased production of toxic intermediates in the univalent reduction of oxygen such as $H_2O_2$, $O_2^-$, and OH · . With the amplified presence of these toxic oxygen species, the cell would need to mount a defense against them. The induction of CA and/or SOD could be one possible defense. The results for *L. monocytogenes* in this experiment were similar (Fig. 1 and 2), showing an initial increase in CA activity at salt concentrations around 3%, followed by a decline in CA activity at the higher salt concentrations tested.

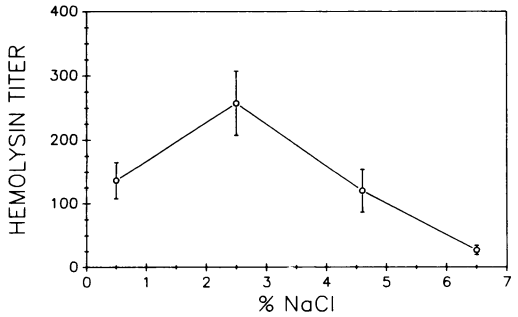


FIG. 3. Thiol-activated hemolysin activity (units per milligram of protein) of *L. monocytogenes* 7644 after growth in TSB containing various levels of NaCl. Error bars represent the standard errors of the means.

SOD, which is an enzyme found in aerobic bacteria and in some anaerobic bacteria, detoxifies $O_2^-$. A number of investigators have found that when cells of SOD-producing bacteria are exposed to increased levels of $O_2$, induction of manganese-containing SOD and, to a lesser extent, CA occurs (22, 23, 27). *L. monocytogenes* possesses a manganese-containing SOD that is inducible when exposed to elevated levels of $O_2$ in the growth medium (22). They also found that *L. monocytogenes* lacks an iron-containing SOD. If intermediate salt levels stimulated the respiratory function of *L. monocytogenes*, then it should follow that SOD would be induced, showing enhanced activity. The results found for *L. monocytogenes* LCDC 81-861 SOD activity (Fig. 2) correlate well with this hypothesis. *L. monocytogenes* 7644 SOD activity follows this theory as well, except for an unexplained reproducible increase in activity in cells cultured in TSBS 6.5 (Fig. 1).

Hemolysin-producing strains of *L. monocytogenes* become hyperhemolytic under certain conditions. The hemolytic activity may be enhanced by maintenance at reduced temperatures for a prolonged period (5, 11, 15). Some overnight cultures of *L. monocytogenes* became hyperhemolytic when briefly incubated at 56°C (15). The hemolytic titer unexplainedly increased when a strain was propagated in charcoal-treated broth (9). The only chemical stimulus found in the literature for hemolysin production by *L. monocytogenes* was lowering of the iron content in the growth medium (3). When 3% NaCl was added to growing cultures of *S. aureus*, the exonuclease production increased fivefold, whereas the activity was augmented by fourfold in 1% NaCl (29). The results for TAH activity by *L. monocytogenes* 7644 (Fig. 3) in this experiment correspond well with the findings for staphylococcal exonuclease. Since listeriolysin is an exoenzyme, it may be that the intermediate NaCl concentration would allow for more efficient transport across the membrane or the permeation of an inducer into the cell.

*L. monocytogenes* 7644 is more heat resistant than *L. monocytogenes* LCDC 81-861 (4). The CA and SOD activities drop at a greater rate for *L. monocytogenes* LCDC 81-861 subjected to sublethal heat injury compared with the rate for *L. monocytogenes* 7644 (4). Knabel et al. (16) found that *L. monocytogenes* F5069 exhibited greater heat resistance with added CA and SOD after heat injury when incubated aerobically. The greater extent of heat injury to *L. monocytogenes* LCDC 81-861 grown in TSBS 4.6 and TSBS 6.5 could be due to a higher rate of inactivation of CA and SOD, leading to an increase in toxicity exhibited by $O_2^-$ and $H_2O_2$ in the recovery medium. Alternatively, it may be that *L. monocytogenes* LCDC 81-861 is near the limit of its heat resistance. Weakening of the membrane of cells grown in osmotically stressful conditions such as TSBS 4.6 and TSBS 6.5 might cause enhanced heat lability.

The findings of this study may increase concern about the possibility of enhanced virulence of *L. monocytogenes* in foods with a salt content of approximately 1 to 3%. Shaha-

mat et al. (25) found a decrease in inhibitory action by NaCl on *L. monocytogenes* when stored at 4°C. An increase in virulence by some *L. monocytogenes* strains kept at 4°C has been observed (5, 30). These findings coupled with the results of this study might explain the *L. monocytogenes* septicemia of the elderly man who ate salted mushrooms stored at cold temperature (12). Thus, particular caution may need to be taken in food industries that deal with moderately salted foods stored at cold temperatures under aerobic conditions.

## ACKNOWLEDGMENTS

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